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Full Length Research Paper

Simultaneous determination of three active constituents in *Erigeron breviscapus* by high-performance liquid chromatography

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A rapid, simple and reliable high-performance liquid chromatographic (HPLC) method was developed for simultaneous determination of compound 1 (scutellarin), compound 2 [1,5-dicaffeoylquinic acid (1,5-DQA)] and compound 3 [1,3-dicaffeoylquinic acid (1,3-DQA)] in *Erigeron breviscapus*. The three constituents were measured on a Zorbax XDB-C_{18} column (4.6 mm × 150 mm, 5 µm) with a gradient elution of acetonitrile (0.5%) aqueous phosphoric acid at wavelength of 335 nm and a flow rate of 1.0 ml/min. Linearity of each standard was established in the concentration range of 19.375 to 310.0 µg/ml for compound 1, 6.125 to 98.0 µg/ml for compound 2, and 3.875 to 62.0 µg/ml for compound 3, respectively, with correlation coefficient $r > 0.9990$. Average recoveries ($n = 6$) of compounds 1 to 3 were 100.3% with a relative standard deviations (RSD) of 1.5%, 98.9% with a RSD of 1.9%, 98.6% with a RSD of 2.0%, respectively. The proposed method was successfully applied to simultaneously determine the three active constituents in *E. breviscapus* from different habitats and germplasm resources for the first time.

Key words: high-performance liquid chromatography (HPLC), *Erigeron breviscapus*, scutellarin, 1,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid.

INTRODUCTION

Erigeron breviscapus (Compositae) known as "Dengzhanxixin", is mainly distributed in southwest of China, especially in Yunnan Province (Institutum Botanicum Kunmingense, Academiae Sinica, 1975). The whole plant was reported to be rich in active flavonoids, flavone glycosides, polyphenolics (Chen, 2002; Zhang, 2000, 2001, 2007; Yue, 2000; Gao, 2006), and is traditionally used as an important herbal drug to treat, for example coronary heart disease, anginapectoris, cerebral infarction, and chronic arachnoiditis, or to expel cold, and relieve exterior syndrome (Gali, 1991; Prochaska, 1992; Liu, 2002, 2008; Gao, 2007).

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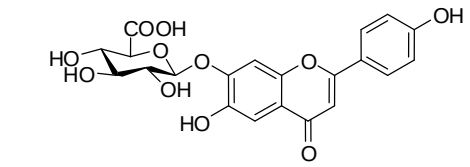
In addition, the ethanolic extract of *E. breviscapus* showed antibacterial, and antifungal properties (Liu, 2003). Accordingly, the plant of *E. breviscapus*, used as an important medicinal resource is widely cultivated in Yunnan province, China. Although Scutellarin, a flavone glucuronide, as a major effective constituent with significant pharmacieutical activity in *E. breviscapus*, have been clinically used to treat cardiocerebral vascular diseases (Li, 2011; Deng, 2002), polyphenolic compounds including 1,5-dicaffeoylquinic acid (1,5-DQA) and 1,3-dicaffeoylquinic acid (1,3-DQA), contained in *E. breviscapus* in a large amount, are drawing particular interests due to free-radical scavenging, anti-human immune deficiency virus (HIV), and platelet aggregation inhibitory activity (Yang, 2005, 2006; Gu, 2007; Danino, 2009; Slanina, 2001).

The extensive and potent pharmacological activities of



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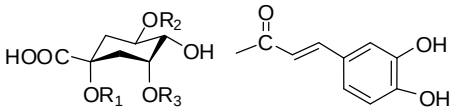

caffeoyl

	R_1	R_2	R_3
(2)	caffeoyl	caffeoyl	H
(3)	caffeoyl	H	caffeoyl

Figure 1. Molecular structures of compounds 1 to 3 (1 = scutellarin, 2 = 1,3-dicaffeoylquinic acid, 3 = 1,5-dicaffeoylquinic acid).

metablites in *E. breviscapus* have prompted the investigation of this plant, using analytical methods such as ultra violet radiation (UV) (He, 2010; Dong, 2007), Liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Qu, 2001a, b), CE-ED (Chu, 2005), Electrospray ionization quadrupole time-of-flight tandem mass spectrometry (RRLC-ESI-MS^n/Q-TOF) (Zhang, 2010), Liquid chromatography-diode array detector-electrospray ionization/mass spectrometry (LC-DAD-ESI-MS^n) (Wang, 2010), and high performance liquid chromatography (HPLC) (Zhang, 2002; Wang, 2007; Wang, 2009). However, only severval papers are known regarding the quantitative methods of flavonoids and phenolic acids in *E. breviscapus* or its extract injection (Chu, 2005; Wang, 2007, 2009, 2010).

Moreover, there was no report about simultaneous analysis of compounds 1 to 3 in *E. breviscapus*. Thus, it is essential to establish a simple and efficient analytical method to determine these active compounds, in order to control the quality and optimize fine germplasm resources and ideal cultivated regions of *E. breviscapus*. In this work, we successfully developed a rapid, simple and reliable HPLC method to simultaneously determine a flavonoid, scutellarin, and two phenolic acids, 1,5-DQA and 1,3-DQA in *E. breviscapus* from different habitats and germplasm resources for the first time.

MATERIALS AND MATHODS

Reagents and materials

Materials of *E. breviscapus* were obtained from different places of Yunnan and Guizhou province, China. All these samples were identified by one of the authors Prof. Shengchao Yang (Institute of Chinese Medicinal Materials, Yunnan Agricultural University). The reference compounds 1 and 3 were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China (batch number: 110842-200605, 111717-200501, respectively), and compound 2 was obtained from State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese academy of sciences. The purity of the three reference compounds was determined to be above 98% by HPLC analysis. Their structures were displayed in Figure 1. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); Deionized water was purified by Milli-Q system (Bedford, USA). Phosphoric acid, analytical grade methanol and ethanol were purchased from Tianjin Wind Ship Chemical Reagent Technology Co., LTD (Tinjing, China).

Standard solutions

Standard stock solutions containing 3.1 mg/m scutellarin, 9.8 mg/ml 1,5-DQA, and 6.2 mg/ml 1,3-DQA, respectively, were prepared by dissolving each reference compound in methanol. The working standard solutions with five different concentrations were obtained by further diluting the solutions, respectively. The standard stock and working solutions were stored at 4°C.

Sample preparation

The whole herb of *E. breviscapus* was dried at room temperature, and then ground into fine powder (0.3 g) by a pulverizer, which was extracted with 10 ml 50% methanol (*v/v*) for 30 min in an ultrasonic bath at 25°C. The lost solution was supplied by adding 50% methanol. The extract solution was filtered by a quantitative filter. The filtrate (20 µl) was injected into the HPLC system for analysis.

Chromatographic conditions

Sample detection was carried out using an Agilent 1200 liquid chromatography system equipped with a vacuum degasser, a quaternary pump, a manual injector, a ultraviolet detector (UVD), and a Zorbax XDB-C18 column (150 × 4.6 mm I.D., 5.0 µm particle size). HPLC system for analysis was set as follows: the column temperature at 30°C; the wavelength at 335 nm; acetonitrile (A) and 0.5% phosphoric acid aqueous solution (B) as mobile phase using a gradient elution with 0 to 10% A at the first 5 min, 10 to 15% A at 5 to 7 min, then 15 to 22% A at 7 to 20 min; a flow rate at 1.0 ml/min. The HPLC system was controlled by a HPLC Chemstation.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

In order to achieve a satisfactory separation of multiple components in *E. breviscapus* in a short period, a gradient elution for compounds 1 to 3 was applied. In addition, there were several phenolic hydroxyl and carboxyl groups in their structures (Figure 1), so phosphoric acid was selected to be added in the mobile phase.

Therefore, mobile phase, 0.5% (*v/v*) phosphoric acid aqueous solution and acetonitrile was finally optimized as the key HPLC parameter step by step, and all three compounds could be eluted with low pressure of column

and baseline separation in 20 min. The monitoring wavelength was set on the basis of absorption maxima of the three compounds at 335 nm. Based on these results, we established the optimal separated conditions corresponding to chromatograms of the authentic standards and *E. breviscapus* as described in Figure 2.

Optimization of sample extraction conditions

The univariate approach was applied to optimize the key sample extraction conditions, and extraction solvent and time of compounds 1 to 3 were used as the evaluation markers of extraction efficiency. The extraction solvents including water, methanol (100, 70, 50 and 30%) and ethanol (100, 70, 50 and 30%) were evaluated for their efficiency in extracting the three compounds in *E. breviscapus*. As a result, the peak areas of all these compounds reached the highest values when 50% methanol was used. Thus, the optimum solvent was finally chosen as 50% methanol. Additionally, 50% methanol in an ultrasonic bath at 15, 30, 45 and 60 min were employed to compare their extraction efficiency, respectively. The corresponding peak areas of all analyses were detected to be highest when extraction time was set at 30 min. Finally, the optimal extraction time was selected as 30 min.

Linearity of calibration curves

Acceptable calibration curves were constructed by using standard solutions of the three reference compounds 1 to 3 at five different concentrations. The regression equations corresponding to the analytical constituents along with correlation coefficients, relative standard deviations (RSDs) and linear ranges were established as shown in Table 1.

Precision

The tested samples (n = 6) were injected into the HPLC instrument under the selected optimum conditions. Precision test of the method revealed that RSDs of compounds 1 to 3 were 1.0, 1.5 and 1.9%, respectively. The results were within the acceptable criteria for precision.

Repeatability

The parallel tested sample solutions (n = 6) were prepared for the repeatability test, and then measured by the proposed method. The RSDs of compounds 1 to 3 were confirmed to be 2.4, 1.7 and 2.0%, respectively, which indicated that this method had good repeatability.

Stability of the solution

The test sample was injected into the HPLC instrument once every two hours, within 24 h. The stability test of the method showed that the RSDs of compounds 1 to 3 were 0.7, 1.1 and 1.6%, respectively. Thus, the test sample solutions were proved to be stable in 24 h.

Recovery

Authentic standard solutions were added into the samples containing marker compounds 1 to 3 with known contents, and then recovery experiments were tested. The results showed that the recoveries were 100.3% with a RSD of 1.5% for compound 1, 98.9% with a RSD of 1.9% for compound 2 and 98.6% with a RSD of 2.0% for compound 3, respectively (n = 6).

Contents of the three compounds in germplasm resources of *E. breviscapus*

Investigation of the contents of compounds 1 to 3 in *E. breviscapus* from different germplasm resources by the proposed method led to the results that content of compound 1 was much higher than that of compounds 2 and 3, and contents of 1 to3 in samples of D21 (sourced from Yongren, Yunnan), D1 (sourced from Shuangbo, Yunnan) and D20 (sourced from Wudian, Yunnan) were highest, respectively (Table 2). Moreover, the samples of D20 and D21 showed relatively higher contents for all compounds 1 to 3. Thus, the three samples could be optimized as ideal germplasm resources for cultivation. In addtion, the coefficients of variation (CV) for compounds 1 and 2 were shown to be 47.10 and 56.29% (Table 2), respectively, suggesting that their contents were closely related to genetic diversity, and quality control of *E. breviscapus* with compound 1 would not be effective.

Contents of the three compounds in *E. breviscapus* from different habitats

As shown in Table 3, content variation of compounds 1 to 3 in *E. breviscapus* from the listed regions was found to be of significant difference, which might be due to different growing environments. Samples of NO.Y5, Y7 and Y11 showed highest contents for compounds 1 to 3, respectively, indicating that regions Chuxiong, Tengchong and Jianchuan could be ideal cultivation areas for *E. breviscapus*.

Conclusion

The proposed HPLC method was evaluated in terms of

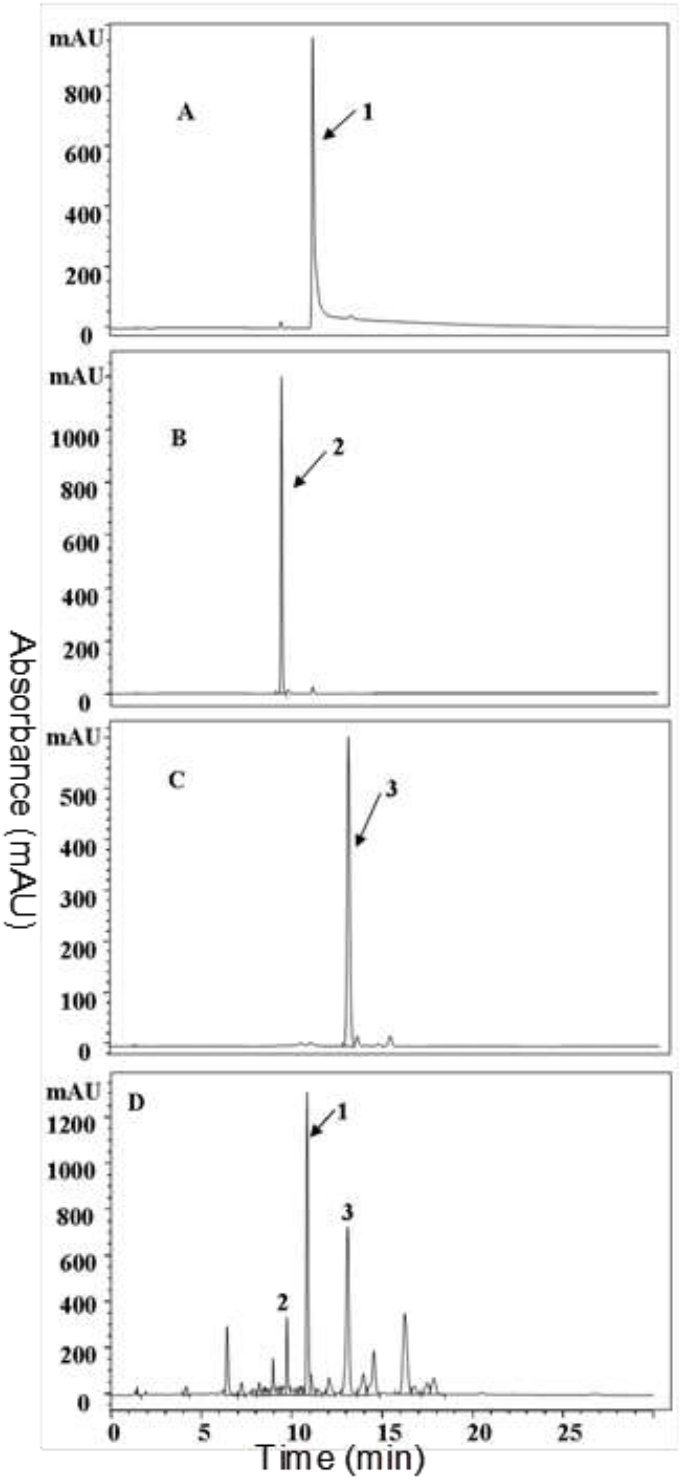

Figure 2. HPLC chromatograms of reference compounds 1 (A), 2 (B), 3 (C) and *E. breviscapus* (D) (1 = scutellarin, 2 = 1,3-dicaffeoylquinic acid, **3** = 1,5-dicaffeoylquinic acid).

linearity, precisions, repeatability, stability and recovery, the results indicated that this method was precise, accurate and reliable for the qualitative and quantitative analysis of the three compounds in *E. breviscapus*. Contents of the three constituents in *E. breviscapus* from different habitats and germplasm resources were success-

Table 1. Linear relationship between peak area and concentration (*n* = 6).

Compound	Regression equation	Linear range (μg/ml)	r
1	Y = 218.3907+40.8941X	19.375~310.0	0.9999
2	Y = 294.8861+62.567X	6.125~98.0	0.9996
3	Y = 707.2223+57.1656X	3.875~62.0	0.9994

X = concentration of the analytes (μg/ml), Y = peak area, r = correlation coefficient

Table 2. Contents of the three maker constituents in germplasm resources of *E. breviscapus* (mg/g) and their coefficients of variation (CV %).

S/N	Source[a]	Compounds		
		1	2	3
D1	Shuangbo, Yunnan	1.495±0.032	0.363±0.008	0.167±0.002
D2	Wuding, Yunnan	2.180±0.011	0.110±0.005	0.132±0.004
D3	Daipu, Yunnan	2.234±0.063	0.155±0.003	0.139±0.001
D4	Songshan, Yunnan	1.458±0.008	0.0875±0.001	0.125±0.000
D5	Xikui, Yunnan	0.972±0.007	0.351±0.001	0.172±0.001
D6	Xuanwei, Yunnan	1.865±0.045	0.0971±0.002	0.128±0.001
D7	Ludian, Yunnan	0.264±0.009	0.0705±0.002	0.0927±0.002
D8	Ludian, Yunnan	1.078±0.008	0.0755±0.0004	0.114±0.001
D9	Zhanyi, Yunnan	1.175±0.019	0.0706±0.001	0.115±0.000
D10	Zhanyi, Yunnan	1.231±0.038	0.156±0.003	0.149±0.002
D11	Zhanyi, Yunnan	1.066±0.024	0.0627±0.001	0.112±0.001
D12	Malong,Yunnan	0.612±0.028	0.0949±0.003	0.0928±0.001
D13	Songming, Yunnan	1.408±0.026	0.260±0.004	0.128±0.001
D14	Songming, Yunnan	2.690±0.026	0.145±0.002	0.137±0.001
D15	Fanjingshan, Guizhou	1.167±0.018	0.145±0.002	0.139±0.001
D16	Yinjiang, Guizhou	2.545±0.029	0.178±0.002	0.140±0.001
D17	Leijiang, Guizhou	2.145±0.542	0.150±0.005	0.132±0.002
D18	Leigongshan, Guizhou	0.598±0.0049	0.141±0.0019	0.121±0.0005
D19	Leishan, Guizhou	2.142±0.026	0.158±0.002	0.125±0.001
D20	Wudian, Yunnan	1.743±0.016	0.323±0.004	0.195±0.002
D21	Yongren, Yunnan	3.371±0.064	0.148±0.002	0.168±0.002
D22	Gucheng, Yunnan	0.683±0.027	0.0567±0.0011	0.104±0.001
D23	Gucheng, Yunnan	1.580±0.023	0.158±0.002	0.115±0.001
Coefficients of variation (CV%)		47.101[b]	56.290[b]	18.660[b]

Each content value represents the mean ± standard deviation (SD) (*n* = 6).[a]Samples of *E. breviscapus* were all obtained from the same cultivation area in Kunming, China, which were grown in the listed regions before.[b]The listed values represents coefficients of variation (CV %) for compounds 1 to 3, respectively.

Table 3. Contents (mg/g) of the three compounds in *E. breviscapus* from different habitats and their coefficients of variation (CV%).

S/N	Source[a]	Compounds		
		1	2	3
Y1	Shangri-La, Yunnan	0.816±0.023	0.359±0.004	0.107±0.002
Y2	Dali, Yunnan	1.347±0.010	0.547±0.004	0.208±0.002
Y3	Dali, Yunnan	1.116±0.0034	0.491±0.0018	0.148±0.001
Y4	Dali, Yunnan	1.505±0.004	0.540±0.002	0.211±0.001
Y5	Chuxiong, Yunnan(cultivated)	1.844±0.002	0.665±0.0037	0.221±0.001

Table 3. Contd.

Y6	Gejiu, Yunnan	1.148±0.009	0.630±0.0041	0.165±0.001
Y7	Tengchong, Yunnan	1.364±0.026	0.834±0.017	0.112±0.0021
Y8	Ludian, Yunnan	1.558±0.016	0.521±0.003	0.177±0.001
Y9	Mile, Yunnan (cultivated)	0.841±0.008	0.707±0.006	0.145±0.001
Y10	Jianchuan, Yunnan	1.408±0.033	0.423±0.0014	0.116±0.001
Y11	Jianchuan, Yunnan (cultivated)	3.862±0.068	0.601±0.0087	0.176±0.001
Y12	Luxi, Yunnan	2.217±0.044	0.491±0.009	0.140±0.002
Y13	Luxi, Yunnan (cultivated)	1.742±0.018	0.160±0.001	0.147±0.001
Coefficients of variation (CV%)		23.728[b]	49.068[b]	31.294[b]

Each content value represents the mean ± SD (n = 6).[a]Samples of *E. breviscapus* were obtained from different regions. [b]The listed values represents coefficients of variation (CV %) for compounds 1 to 3, respectively.

fully determined by the proposed method, which suggested that this alalytical method may be a useful tool to control the qualities of *E. breviscapus* and its injection, and to optimize fine germplasm resources of *E. breviscapus* and ideal cultivated regions.

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